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CAT Reporter Systems: New pCAT®3 Reporter Vectors and Antibodies Provide Increased Expression and Detection Capabilities

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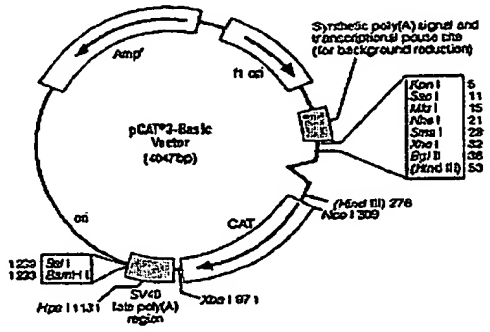
Promega's new pCAT®3 Reporter Vectors and Affinity Purified Anti-CAT antibodies expand the capabilities of chloramphenicol acetyltransferase (CAT) reporter systems. Increased expression of the CAT gene and better cloning versatility, along with highly specific, affinity purified anti-CAT antibodies, add a new level of detection and convenience to studies using the CAT reporter gene.

Introduction

Reporter vectors serve as tools in the analysis of gene expression and regulation. Most frequently, reporter genes have been used for quantitative analysis of factors that regulate mammalian gene expression. These factors may be *cis*-acting, such as promoters and enhancers, or *trans*-acting, such as various DNA-binding proteins. In addition to studying transcriptional gene regulation, vectors with reporter genes have been designed to monitor processes which include *in vivo* and *in vitro* gene delivery systems, protein-protein interactions (1), recombination events (2), gene targeting (3), RNA processing (4), signal transduction (5,6) and transfection efficiency. To facilitate such experiments, Promega provides tools and reagents for the convenient manipulation, expression, and detection of reporter genes in mammalian cells. The available reporter vectors include the pGL2 (Cat.# E1641, E1621, E1631, E1611) and pGL3 Luciferase Reporter Vectors (Cat.# E1741, E1771, E1761, E1751), the pSV-beta-Galactosidase Control Vector (Cat.# E1081), and the pCAT® Reporter Vectors (Cat.# E1041, E1021, E1031, E1011). Here we describe improvements on the design of the pCAT® Reporter Vectors to yield the pCAT®3 Reporter Vectors. These new reporter vectors have been optimized for increased CAT expression and use convenience. Additionally, we present data demonstrating various applications of new, highly specific, affinity-purified anti-CAT antibodies. These antibodies detect CAT in Western blot, immunocytochemistry and ELISA formats, providing greater sensitivity and versatility to CAT reporter gene analysis.

pCAT®3 Reporter Vector enhancements

Except for the presence of SV40 promoter and enhancer elements, the four pCAT®3 Reporter Vectors are identical. The distinguishing features of each plasmid are presented in Figure 1. The design of the pCAT®3 Reporter Vectors increases both CAT expression and user versatility compared to the pCAT® Reporter Vector series. The improvements, described in detail below, are summarized in Table 1.



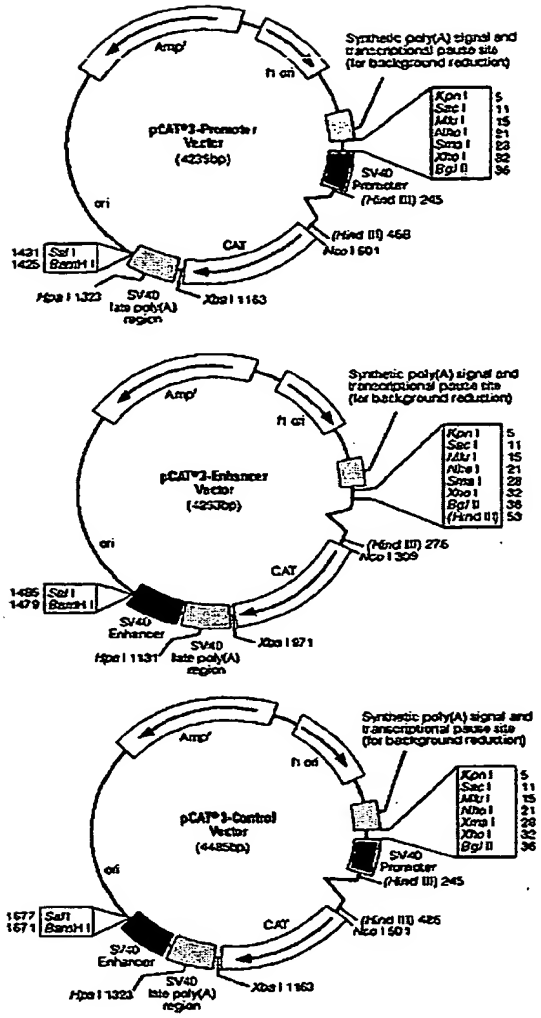


Figure 1. The pCAT®3 Vector circle maps. Additional description: _^_; position of intron; CAT, the chloramphenicol acetyltransferase gene; Ampr, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of plasmid replication in *E. coli*. Arrows within CAT and the Ampr gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA synthesis. Restriction sites shown in parentheses are not unique sites.

Table 1. Characteristics of the pCAT®3 Vectors Compared to the pCAT® Vectors.

Change from pCAT® Vector	Purpose of Modification	Reference
The SV40 small-t antigen intron replaced with a chimeric intron 5′ of the CAT gene.	Intron from SV40 small-t antigen can reduce expression when placed 3′ of certain genes due to cryptic splicing.	(7,8)
Poly(A) signal for CAT changed from early to late SV40 poly(A) signal.	Late SV40 poly(A) signal is more efficient than early SV40 poly(A).	(16)
A synthetic poly(A) and transcriptional pause site was inserted 5′ of the CAT gene and multiple cloning sites.	Reduces background CAT expression while avoiding possible recombination between two SV40 poly(A) sequences in the same plasmid.	(17,18)
Kozak consensus sequence created at the 5′ end of the CAT gene.	Provides for optimal translation efficiency.	(19)

Multiple cloning sites changed.	Increases convenience and provides compatibility with the pGL3 Vectors.
f1 ori included in the vector backbone.	Provides the ability to generate ssDNA for sequencing or mutagenesis.
Nco I site in Kozak sequence made unique.	Increased convenience; facilitates subcloning.
Unique *Xba* I site created just downstream of the CAT gene.	Increased convenience; facilitates subcloning.
*Eco*R I site removed from within the CAT gene (results in the amino acid change Phe73-->Leu73).	Increased convenience; facilitates subcloning.

Chimeric intron 5′ of the CAT gene

An increased level of CAT expression, along with a low background, allows for easier monitoring of transcriptional activity in transfected eukaryotic cells. Many eukaryotic expression vectors, including the pCAT® and pGL2 Reporter Vectors, contain the SV40 small-t antigen intron placed 3′ of the reporter gene with the intent of enhancing gene expression. However, the small-t antigen intron placed downstream of the CAT (7) or beta-galactosidase (8) reporter genes has been observed to decrease expression up to 10-fold due to aberrant splicing occurring between this intron and the sequence of interest (7). To optimize expression of transcripts, a new chimeric intron has been developed. This 132 base pair intron contains the 5′ splice donor site from a human beta-globin intron and the branch point and 3′ splice acceptor site from an IgG intron (9). The sequences of the splice donor, branch and acceptor sites have been optimized to match their respective consensus sequences (10). This intron is also present in Promega's mammalian expression vectors pSI (Cat.# E1721), pCI (Cat.# E1731), and pCI-neo (Cat.# E1841) (11,12). When placed 5′ of the CAT gene, this intron increases CAT expression more than 20-fold in both transient and stable transfections when compared to the same vectors lacking an intron (12). RT-PCR of CAT mRNA transcripts from cells transfected with the pCAT®3-Control Vector generates a single PCR product of the expected size, indicating proper splicing of the chimeric intron (data not shown). For those researchers who would prefer no intron, or would like to experiment with other introns and/or intron locations, the chimeric intron is flanked by *Hin*d III and *Not* I sites for easy removal.

Polyadenylation signals

A poly(A) signal located in the 3′ untranslated region of an mRNA directs the addition of 200-250 adenylate residues to the 3′-end of the RNA transcript (13), enhancing RNA stability and translation (14,15). The SV40 late poly(A) signal, which is included in the pCAT®3 Reporter Vectors, has been shown to be 5-fold more efficient at generating high levels of steady state mRNA than the SV40 early poly(A) signal (16) which is carried by the pCAT® Reporter Vectors.

Spurious transcription originating from cryptic promoter sequences within a vector backbone can lead to background reporter expression, which decreases the sensitivity of reporter gene assays. Such unwanted background expression can be reduced by placing an additional poly(A) signal 5′ of the transcription unit (17). In the pCAT®3 Reporter Vectors we have placed a relatively short, synthetic poly(A) signal upstream of the multiple cloning site. This sequence is based on the highly efficient poly(A) signal of the rabbit beta-globin gene (18). The upstream poly(A) site has little homology to the SV40 late poly(A) site, thus minimizing the chances of recombination. In addition, stop codons were placed in all three reading frames upstream of the multiple cloning site to further reduce background at the translational level. Although every attempt has been made to design the pCAT®3 Reporter Vector backbones to be transcriptionally neutral in eukaryotic cells, any vector may contain unexpected regulatory sites which could exhibit undesirable activities in different cell types. Therefore, it is important that the proper vector controls always be used.

Enhancements in versatility

A Kozak consensus sequence (GCCGCCACCATG) has been placed at the 5′ end of the CAT gene to increase the efficiency of translation initiation (19). This sequence maximizes CAT expression and provides a convenient *Nco* I site at the 5′ end of the CAT gene. To make this *Nco* I site unique, the other *Nco* I sites within the enhancer, promoter, and CAT gene have been removed. Additionally, an *Eco*R I site has been removed from within the CAT gene and a unique *Xba* I site created at the 3′ end of the CAT gene to facilitate subcloning.

The multiple cloning sites of the pCAT®3 Reporter Vectors (Figure 2) are identical to those in the pGL3 Reporter Vectors (20). The orientation of these sites allows for nested deletion analysis using Exonuclease III (e.g., Promega's Erase-a-Base® System, Cat.# E5850). A conveniently located *Sma* I site allows blunt-ended DNA fragments to be inserted into the upstream multiple cloning site. Since blunt-ended ligation often destroys the restriction site, flanking restriction enzyme sites can be used for subsequent subcloning or

analysis. Two unique cloning sites, *Bam*H I and *Sal* I, located downstream of the transcribed region, provide convenient cloning sites for cis-acting mediators such as enhancers, or for inserting selectable markers (e.g., the *neo* gene) to generate stable cell lines. Moreover, the overhanging ends created by *Bum*H I and *Sal* I cleavage of these sites are compatible with the upstream *Bgl* II and *Xho* I sites, respectively, providing for positional analysis of inserted DNA. Note that two primers, RVprimer3 and RVprimer4 (RV for reporter vector) are also available for sequencing cloned inserts.

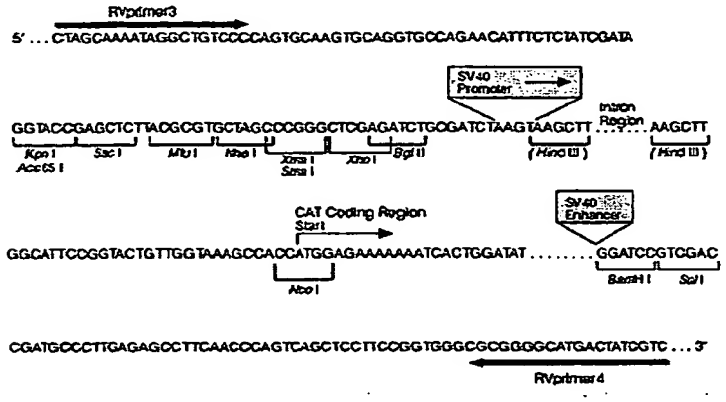


Figure 2. The pCAT®3 Vector multiple cloning regions. Shown are the upstream and downstream cloning sites and the locations of the sequencing primers, RVprimer3 and RVprimer4 (RV: reporter vector). The large primer arrows indicate the direction of sequencing. The positions of the promoter (in the pCAT®3-Promoter and pCAT®3-Control Vectors) and the enhancer (in the pCAT®3-Enhancer and pCAT®3-Control Vectors) are shown as insertions into the sequence of the pCAT®3-Basic Vector. (Note that the promoter replaces four bases [AAGT] of the pCAT®3-Basic Vector.) The sequence shown is of the DNA strand generated from the f1 origin.

Performance of pCAT® and pCAT®3 Reporter Vectors

We have observed a 5- to 10-fold increase in expression of CAT in HeLa cells from the pCAT®3-Control Vector when compared to the pCAT®-Control Vector (Figure 3). However, the observed level of CAT expression will vary with the cell type and the transfection conditions. While the level of CAT expression in HeLa cells transfected with the pCAT®3-Control Vector is significantly increased, the background level of expression from the pCAT®3-Basic, -Enhancer and -Promoter Vectors remains low. Figure 4 shows representative data comparing the performance, in HeLa cells, of the four pCAT®3 Reporter Vectors to their pCAT® Reporter Vector counterparts.

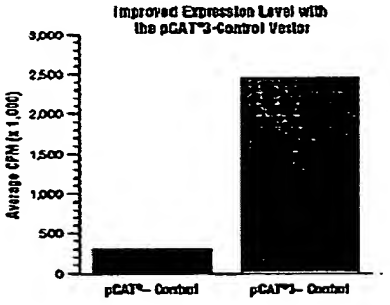


Figure 3. Comparison of CAT activities expressed in HeLa cells transfected with the pCAT®-Control and pCAT®3-Control Reporter Vectors. One day before transfection, 1 x 10^5 HeLa cells were seeded into 60mm culture plate wells in DMEM + 10% fetal bovine serum (FBS). In duplicate experiments, the cells were transfected with 5µg of the indicated plasmid DNA using the calcium phosphate method of the ProFection® Mammalian Transfection System (Cat.# E1200) (21). Following an overnight incubation, the medium was replaced with fresh DMEM + 10% FBS and the cells allowed to recover 48 hours before they were harvested using 400µl/well 1X Reporter Lysis Buffer. CAT activity in cellular extracts was measured by liquid scintillation counting using 50µl of each extract and the CAT Enzyme Assay System with Reporter Lysis Buffer (Cat.# E1000) as described (22).

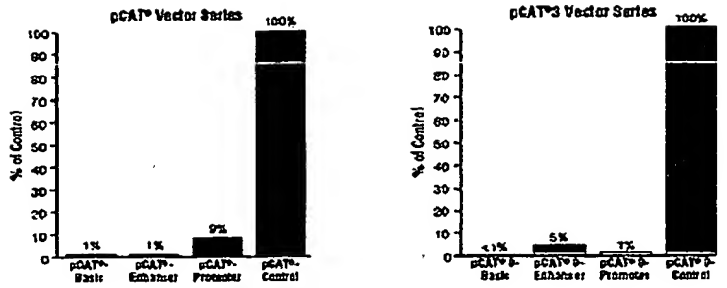


Figure 4. CAT activities expressed in HeLa cells transfected with the pCAT® and pCAT®3 Vector series. Cells were transfected with the indicated vectors and assayed as described in Figure 3.

Affinity purified anti-CAT polyclonal antibodies

Many reporter proteins can be quantitated by their catalytic activities using enzyme specific assays. These assays are often very sensitive because the reporter enzyme can be present at very low levels and still generate a measurable signal in the assay. However, it is often advantageous to detect reporter expression using reporter-specific antibodies. Chicken polyclonal antibodies (pAb) against the CAT protein were affinity purified (23) to obtain a highly specific preparation of Anti-CAT pAb. These antibodies allow for the detection of CAT expression in a variety of common immunological formats, including Western blot, immunocytochemistry and ELISA (data not shown).

In immunoblotting (24,25), antibodies detect proteins in both active and inactive forms, or in full length or proteolytically processed forms. SDS-polyacrylamide gel electrophoresis (26), followed by blotting and binding with specific antibodies, allows visualization of the specific forms of the protein. An example of immunoblotting with the new Affinity Purified Anti-CAT pAb is shown in Figure 5.

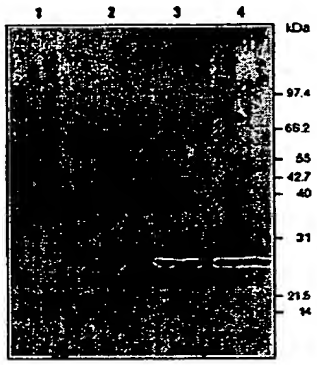


Figure 5. Western blot analysis using Affinity Purified Anti-CAT pAb. One day before transfection, 3 x 10^5 CHO cells were seeded into 60mm culture plate wells in F12 Medium + 10% FBS. The cells were transfected with 5µg pCAT®3-Control DNA using the calcium phosphate method of the ProFection® Mammalian Transfection Systems (21). Following an overnight incubation, the medium was replaced with fresh medium and the cells allowed to recover before they were harvested using 400µl/well of 1X Reporter Lysis Buffer and three freeze/thaw cycles. Lanes: Lane 1, 10µl untransfected CHO cell extract (~6µg total protein); Lane 2, 10µl cell extract from pCAT®3-Control Vector-transfected CHO cells (~3µg total protein); Lane 3, 5ng of purified CAT (Cat.# E1051); Lane 4, 20ng of purified CAT.

Immunostaining of cells in a culture dish or on a slide is a common application for reporter antibodies (25). When antibodies are used to stain cells or tissues, they can be used to determine not only the presence of an antigen, but also its subcellular localization. Cell staining is also a convenient way to determine the percentage of cells that have been transfected with reporter DNA. Figure 6 illustrates CAT-transfected COS cells detected by immunostaining with the Promega Affinity Purified Anti-CAT pAb.

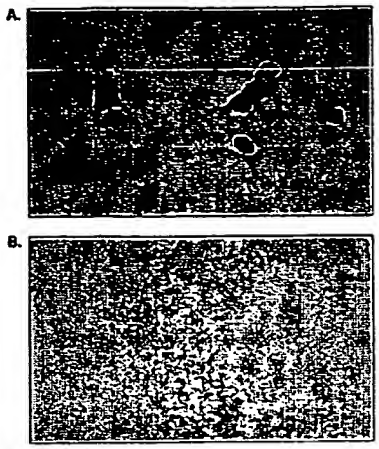


Figure 6. Immunocytochemistry using Affinity Purified Anti-CAT pAb. The Affinity Purified Anti-CAT pAb detects CAT protein specifically in cells transfected with a vector expressing CAT. COS cells were transfected with a CAT expression vector **(Panel A)** or beta-galactosidase expression vector **(Panel B)** and stained with the Affinity Purified Anti-CAT pAb. COS cells were grown on coverslips in multi-well plastic culture dishes and transfected with the indicated vector using calcium phosphate transfection. Following a brief PBS wash, the cells were fixed in 4% paraformaldehyde in PBS for 5 minutes. Cells were permeablized with 0.3% Triton® X-100 in PBS for 10 minutes. The Affinity Purified Anti-CAT pAb was diluted to 750ng/ml in 1% normal goat serum in PBS and allowed to bind overnight. The primary pAb was visualized using a 1:200 dilution of biotinylated goat anti-chicken secondary antibody followed by incubation in 1:50 dilution of VECTASTAIN® *ELITE* ABC reagent (Vector Laboratories Cat.# PK-6100) for 1 hour followed by a PBS rinse. Binding was detected by applying DAB (Vector Laboratories Cat.# SK-4100) to the cells for 10 minutes. After a PBS rinse, the cells were dehydrated in ethanol, cleared in toluene and mounted. (Data provided by Dr. P. Baluk, Cardiovascular Research Institute, School of Medicine, UCSF).

Summary

The pCAT®3 Reporter Vectors provide increased CAT expression and greater versatility. The higher level of expression makes these vectors particularly useful in experiments where greater levels of CAT expression are needed (e.g., when studying weak promoter elements). Moreover, the plasmid backbone has been designed to exclude a number of potential transcription factor binding sites and include useful multiple cloning sites upstream and downstream of the CAT reporter gene. Finally, CAT expression can be detected in a variety of immunological formats with new, highly specific Affinity Purified Anti-CAT pAb.

References

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Ordering Information

Product	Size	Cat.#
pCAT®3-Control Vector	20µg	E1851
pCAT®3-Enhancer Vector	20µg	E1881
pCAT®3-Promoter Vector	20µg	E1861
pCAT®3-Basic Vector	20µg	E1871

Product	Size	Cat.#
Anti-CAT-pAb, Affinity Purified	200µl	E4521